# Studying nonobstructive azoospermia in cystinosis: histologic examination of testes and epididymis and sperm analysis in a Ctns / mouse model

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Abstract: OBJECTIVE: To study the pathogenesis of male infertility in cystinosis due to nonobstructive azoospermia, using a Ctns(-/-) mouse model. DESIGN: Observational case-control study. SETTING: Academic research laboratory. ANIMAL(S): Male C57BL/6 Ctns(-/-) mice were compared with C57BL/6 wild-type (wt) mice. INTERVENTION(S): None. MAIN OUTCOME MEASURE(S): Fertility was studied using litter size (n = 3 vs. n = 2). After animals were sacrificed, testes, epididymis, and vas deferens were removed for testicular cystine measurements (n = 5 vs. n = 6), histologic studies (n = 3 vs. n = 3), and sperm analysis (n = 3 vs. n = 3). RESULT(S): Mean testicular cystine content was significantly higher in Ctns(-/-) mice compared with wt mice (26.6 ± 1.22 vs. 0.1 ± 0.01 nmol cystine/mg protein). Testes of Ctns(-/-) mice had lower weight compared with wt mice (0.096 ± 0.009 g vs. 0.112 ± 0.004 g), but mice fertility was similar (litter size 6.6 ± 1.4 vs. 6.3 ± 2.6 pups). Neither histologic nor sperm abnormalities were found. CONCLUSION(S): The Ctns(-/-) mouse model generated on C57BL/6 background is not suitable for clarifying the pathogenesis of male infertility in cystinosis. The etiology of nonobstructive azoospermia in these patients remains unclear.

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# Studying nonobstructive azoospermia in cystinosis: histologic examination of testes and epididymis and sperm analysis in a *Ctns*$^{-/-}$ mouse model


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- Objective
- To study the pathogenesis of male infertility in cystinosis due to nonobstructive azoospermia, using a *Ctns*$^{-/-}$ mouse model.
- Design
- Observational case-control study.
- Setting
- Academic research laboratory.
- Animal(s)
- Male C57BL/6 *Ctns*$^{-/-}$ mice were compared with C57BL/6 wild-type (wt) mice.
- Intervention(s)
- None.
- Main Outcome Measure(s)
- Fertility was studied using litter size (n = 3 vs. n = 2). After animals were sacrificed, testes, epididymis, and vas deferens were removed for testicular cystine measurements (n = 5 vs. n = 6), histologic studies (n = 3 vs. n = 3), and sperm analysis (n = 3 vs. n = 3).
- Result(s)

- Mean testicular cystine content was significantly higher in *Ctns*$^{-/-}$ mice compared with wt mice (26.6 ± 1.22 vs. 0.1 ± 0.01 nmol cystine/mg protein). Testes of *Ctns*$^{-/-}$ mice had lower weight compared with wt mice (0.096 ± 0.009 g vs. 0.112 ± 0.004 g), but mice fertility was similar (litter size 6.6 ± 1.4 vs. 6.3 ± 2.6 pups). Neither histologic nor sperm abnormalities were found.
- Conclusion(s)
- The *Ctns*$^{-/-}$ mouse model generated on C57BL/6 background is not suitable for clarifying the pathogenesis of male infertility in cystinosis. The etiology of nonobstructive azoospermia in these patients remains unclear.



## Key Words

- Cystinosis;
- infertility;
- testis;
- epididymis;
- sperm analysis;
- CASA;
- nonobstructive azoospermia


Cystinosis is a rare autosomal recessive disorder caused by mutations of the *CTNS* gene (17p13), encoding the lysosomal cystine carrier cystinosin (1). This results in intralysosomal accumulation of cystine in all tissues. Patients develop generalized proximal tubular dysfunction called renal Fanconi syndrome during the first year of life and, if left untreated with cysteamine, end-stage renal disease before the age of 10 years (2). Besides renal disease, patients with cystinosis suffer from extrarenal complications involving the eyes, muscles, central nervous system, and various endocrine organs (2). The latter include primary hypothyroidism (3), endocrine (4), exocrine (5), and pancreas insufficiency, delayed onset of puberty (6) and primary hypogonadism in boys, characterized by low T levels and high levels of LH and FSH (7). Recently it was discovered that male patients with cystinosis are infertile due to nonobstructive azoospermia. The pathogenesis of azoospermia in male patients with cystinosis, however, remains unknown (8).

## MATERIALS AND METHODS

For these studies, we examined male Ctns_/_ mice generated on a C57BL/6 background and compared them with C57BL/6 wt littermates. The Ctns_/_ mice were generated by replacing the last four exons of Ctns with an IRES-bgalneo cassette, as previously described (9). First, mixed strain Ctnsþ/_ mice were backcrossed with wt mice to generate congenic mice. Next, the congenic Ctnsþ/_ were mated to obtain Ctns_/_ mice. All mice used in this study were genotyped before the onset of the experiments. All animal experiments were performed in accordance with national guidelines for the care and use of research animals. For testicular cystine measurements, five adult

Ctns_/_ and six wt mice were examined (including the six mice in which sperm analysis was performed). They were sacrificed for noninterventional studies between the age of 8 and 12 months. Testes were surgically removed, snap-frozen in liquid nitrogen, and transported on dry ice. Next, the testes were sonicated until complete disruption in the presence of 10 mM N-ethylmaleimide. The protein fraction was precipitated by the addition of 10% 5-sulfosalicylic acid dehydrate and measured using the Pierce bicinchoninic acid protein assay. Cystine levels were measured by high-performance liquid chromatography (HPLC), as previously described (10). Fertility was studied by counting litter size of pregnancies in female Ctnsþ/_ and Ctnsþ/þ (wt) mice, fathered by either Ctns_/_ or wt mice. In total, three male Ctns_/_ mice and two male Ctnsþ/þ (wt) mice were mated with five female Ctnsþ/_ mice and one female wt mouse. For histologic studies (including weight), three adult Ctns_/_ and three wt mice were included. They were sacrificed for noninterventional studies at the age of 18 months. Testes and epididymis were surgically removed for histologic examinations and were immediately fixed in Bouin's solution. Next, they were embedded in paraffin and 5-mm sections were stained with periodic acid-Schiff (PAS)/hematoxylin for morphological analyses.
For sperm analysis, three adult Ctns_/_ and three wt mice were included. They were sacrificed by cervical dislocation for noninterventional studies at the age of 9 months. Epididymis and vas deferens were surgically removed and placed in 500 mL of Dulbecco's modified Eagle's medium (DMEM) supplemented with 3% bovine serum albumin (BSA; Sigma) on a 37_C plate. The epididymides were gently scraped with needles and the vasa deferentia were pressed to allow sperm to get out. The samples were kept at 37_C and sperm analysis was performed within 60 minutes after collection by computer-aided sperm analysis (CASA). Therefore, cells were analyzed using the Integrated Visual Optical System sperm analyzer (version 10.7, Hamilton-Thorne Biosciences) as described previously (11). Nonprogressive and progressive spermatozoa were included in the analysis; slow spermatozoa were considered nonmotile. In each sample the following motility parameters were analyzed: concentration, total motility, progressive motility (including both straight progressive and nonstraight progressive spermatozoa), energy of motion (measured by average path velocity [VAP], straight-line velocity [VSL], curvilinear velocity [VCL], and beat cross frequency) and the pattern of sperm head motion (measured by linearity, straightness, and the amplitude of lateral head displacement). Hyperactivity was assessed using the criteria of Burkman (linearity <65%, amplitude of lateral head displacement >7.5 mm, and VCL >100 mm/s) (12). The Student's t-test was used for statistical analysis. P values %.05 were considered to be statistically significant.

RESULTS

Mean_ SD testicular cystine content was significantly higher in Ctns_/_ mice compared with wt mice (26.6 _ 1.22 nmol cystine/mg protein vs. 0.1 _ 0.01 nmol cystine/mg protein, P<.01). Fertility in 57BL/6 Ctns_/_ mice was normal compared with wt mice. Mean _ SD litter size was 6.6 _ 1.4 pups versus 6.3 _ 2.6 pups; these were the mean of eight and nine pregnancies, respectively. Female genotype (Ctnsþ/þ or Ctnsþ/_) did not affect litter size. Mean _ SD testes weight in the Ctns_/_ mice was lower compared with wt mice (0.096 _ 0.009 g vs. 0.112 _ 004 g, P¼.05). Although smaller in size, the testes of all three Ctns_/_ mice showed a similar morphology to that of wt mice without detectable abnormalities (Fig. 1A and B). All types of germ cells were present.

Likewise, the epithelium of the epididymis of Ctns_/_ and wt mice showed similar morphology in both caput, corpus (Fig. 1C and D), and cauda. The epididymides were filled with normal sperm. Overall, there were no histologic abnormalities. The results of the sperm analysis are shown in Table 1. Because sperm parameters depend on mouse strain as well as on the criteria that have been set to program the CASA, there are no reference values for the different parameters. Therefore, values of Ctns_/_ mice were compared to those of their wt littermates. There was no difference in either parameter between the two groups.

## DISCUSSION

Infertility is a major problem worldwide, affecting approximately 15% of the reproductive age opulation. In male factor infertility, it is estimated that about 50% of the cases are contributed or caused by genetic abnormalities (13). In several genetic disorders, such as cystic fibrosis or Bardet-Biedl syndrome, longer patient's survival due to advanced medical care revealed male factor infertility as a part of the clinical phenotype. Another example is the lysosomal storage disorder nephropathic cystinosis, which, in the past decades, has changed from a lethal disorder of childhood to a potentially treatable disorder allowing patients to survive into adulthood (14). Although the gonadal damage in cystinosis due to excessive cystine accumulation has been initially supposed to cause infertility, the early use of the cystine-depleting agent cysteamine was believed to counteract this complication and to have a positive effect on other endocrine organs and muscles. Our recent study demonstrated that unfortunately this was not the case as young men with cystinosis had nonobstructive azoospermia, although treated with cysteamine from the time of diagnosis at an early age (8).

The current study aimed at finding the etiology of male factor infertility in patients with cystinosis, using a C57BL/ 6 Ctns_/_ mouse model. Previous studies have shown increased intracellular cystine levels in kidney, spleen, heart, liver, lung, muscle, eye, and brain in Ctns_/_ mice, compared with their wt littermates. Furthermore, these mice showed pronounced histologic lesions of the proximal tubules combined with proximal tubular dysfunction and progressive chronic renal failure (9), resembling the human cystinosis phenotype. Interestingly, the renal phenotype was only observed in Ctns_/_ mice generated on C57BL/6 and not on a mixed 129Sv _ C57BL/6 genetic background (9, 15). In contrast to male patients with cystinosis, we found that C57BL/6 Ctns_/_ mice exhibit a normal fertility compared with wt mice. To exclude subclinical changes in mice fertility, we conducted a comparative histologic study of testes and epididymides of C57BL/6 Ctns_/_ and wt mice littermates, as well as detailed sperm analysis. For practical reasons, sperm analysis was performed with sperm obtained from the epididymis and vas deferens, as opposed to ejaculated sperm that was used to diagnose azoospermia in patients with cystinosis. Although the intracellular cystine content in testes obtained from Ctns_/_ mice was increased, neither histologic examination nor sperm analysis showed any difference compared with wt mice. The low percentage of total and progressive motility of spermatozoa that was found in this study was probably caused by the delay between sperm collection and analysis. In men, it is advised to keep the time between ejaculation and sperm analysis at less than 60 minutes, as it is known that after that time sperm quality starts to decline (16). In our study, all samples were measured within 60 minutes after collection. Furthermore, because the same conditions applied on both samples obtained from Ctns_/_ and wt mice, it can be stated that there was no

statistically significant difference between the groups. The presence of a less severe or even the absence of a human phenotype in knock-out mouse models is not exceptional and has been demonstrated in several other diseases such as the lysosomal storage disorder Pompe disease (17), Gitelman syndrome (18), or L€owe syndrome (19). The differences are mostly attributed to the presence of the genetic modifiers compensating for the absence of the gene of interest in mice. In Gitelman syndrome, the generation of a knock-out mouse model using a different genetic background led to a renal phenotype mimicking the human situation in more detail (20). L€owe syndrome and Dent disease type 2 are caused by mutations in the OCRL1 gene that encodes a type II phosphoinositide 5-phosphatase. A combined knock-out model of Ocrl1 and the replacement of the murine Inpp5b gene by the human INPP5B gene, which encodes another type II phosphoinositide 5-phosphatase, does cause a phenotype that is comparable to human L€owe syndrome, whereas the knock-out of Ocrl1 alone does not (21). In our study using C57BL/6 Ctns_/_ mice the absence of cystinosin led to cystine accumulation in the testes; however, no histologic or functional differences were found. It cannot be excluded that the genetic background of mice played a role, as was demonstrated for the renal phenotype (9, 15). Taken together, in contrast to the renal phenotype that partially mimics the human disease, C57BL/6 Ctns_/_ mice show normal testicular morphology and function. Therefore, this mouse model does not seem helpful to address the pathogenesis of nonobstructive azoospermia in patients with cystinosis.